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(57) Abstract

The present invention relates to antibodies which are capable of being conjugated at specific sites, to processes for the site-directed conjugation of such antibodies, to antibodies that have been conjugated at a specific site and to the use of such antibodies in therapy and diagnosis.

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ANTIBODIES

The present invention relates to antibodies which are capable of being conjugated at specific sites, to processes for the site-directed conjugation of such antibodies, to antibodies that have been conjugated at a specific site and to the use of such antibodies in therapy and diagnosis.

Antibodies are globular proteins which represent a vital component of the mammalian immune response to foreign disease inducing agents. Antibodies may be manufactured *ex vivo* by any of a number of methods, and such antibodies, particularly monoclonal antibodies and/or fragments thereof, have proved valuable as both diagnostic and therapeutic agents. The utility of antibodies stems from their unique antigen specificity, i.e., their ability to chemically recognise and remain bound to discrete chemical moieties such as pathogen antigens or tumour-associated antigens. Another aspect of their utility is their diversity, i.e., the ability of mammals (and now other processes such as phage display) to create a very large variety of discrete, genetically defined antibodies (monoclonal antibodies). A final aspect of their utility is their capacity to interact via their "constant" regions. This latter aspect determines other sets of properties, for example, those properties common to isotypes such as interaction with effector cells, complement or other binding moieties like protein A.

The usefulness of an antibody or fragment may be enhanced by chemically coupling one or more further molecular moieties (referred to herein as substances) either directly or indirectly that convey properties to the conjugate that are not naturally present in the constituents alone. Such properties may include a reporter function such as a dye or a radionuclide, an enzymatic function, a second binding function (such as with biotin-avidin), a drug (such as adriamycin), a cytotoxic function (such as with ricin), a chelator, or a chemical linkage moiety that may, in turn render the antibody capable of subsequent reaction with any of a variety of molecular moieties. Thus an antibody conjugate comprises an antibody and an active substance (substance) which is either directly or indirectly conjugated to the antibody. For example, when the active substance is a radionuclide, the radionuclide may be directly conjugated to the antibody or alternatively it may be indirectly conjugated to the antibody via a chelator such as for example TMT or even a chelator which is in turn linked to the antibody by a further protein reactive group (cross-linker) such as, for example, bromoacetyl. Similarly, the

active substance could be a molecular chimaera for use in enzyme-prodrug therapy, such a chimaera comprises a transcriptional regulatory DNA sequence capable of being activated in a mammalian cell such as a cancer cell and a DNA sequence operatively linked to the transcriptional regulatory DNA sequence and encoding a heterologous enzyme capable of catalysing the conversion of a prodrug which is administered subsequently,into an agent toxic to the cancer cell. This molecular chimera may be directly conjugated to the antibody or alternatively, it may be contained within a viral vector or liposome with the viral vector or liposome then being attached to the antibody (see European application No.90309430.8).

Many means have been described for the chemical addition or crosslinking of molecular entities to antibodies and their fragments, and these reactions are commonly referred to as conjugation reactions. Conjugation reactions exploit the chemical functional groups that occur naturally in the antibody. For example, a common approach is to target primary amines (mainly the ε amino groups of lysine residues). Another common example of this approach includes the iodination (such as ^{125}I) of tyrosine residues. A disadvantage of this approach is that it is random. That is, lysine or tyrosine residues may occur throughout the structure and therefore the natural properties that these residues help convey -- such as, for example, antigen recognition, complement reactivity or effector cell interaction -- may be compromised. The behaviour of the conjugate is the average of the behaviour of all the individual unique components, some of which may be entirely useless or detrimental. The random approach is also known to affect antigen recognition.

Site-directed conjugation, i.e., conjugation to a specific amino acid residue within the antibody structure, would convey the advantage that the resulting conjugate is not a mixture of different products. The properties conveyed by the antibody to the conjugate, such as antigen reactivity or pharmacokinetic stability, can thus be ascribed to a defined chemical structure. Furthermore, the site may be selected so as to be spatially removed from areas known to convey antigen-binding properties. For example, the variable region of an antibody is known to contain the antigen binding site and the CH2 domain of the heavy chain is known to contain effector (FcR) and complement (C1q) interactive residues. Thus, additions to the CH3 domain of the heavy chain may avoid compromising these functions.

Two examples of site-directed conjugation procedures to antibodies have been described. The conjugation of antibodies to the carbohydrate portion of antibodies has been detailed (O'Shannessy and Quarles. *J.Immunol. Met 99:* 153-161, 1987). Human IgG antibodies have one common glycosylation site at asn297 in the CH2 domain. (Note that all antibody amino acid residues described herein are numbered according to the EU index. Kabat, *et al., Sequences of proteins of immunological interest,* 5th ed., NIH publication No. 91-3242, 1991. The common isotypes are referred to as G1, G2 G4 etc.). The glycosylation conjugation procedure presents the risk of altering carbohydrate that might be required for antigen or effector interaction (Lund, *et al.. Molec. Immunmol 27:* 1145, 1990; Isaacs, *et al.. J. Immunol. 148*: 3062, 1992).

Another example of site-directed conjugation to antibodies involves the creation, by site- directed mutagenesis, of a free thiol on the antibody. Antibodies naturally contain cystine residues whose thiol groups are joined by disulfide bridges. The position of the naturally occurring cystine residues is highly conserved among species indicating that these residues are essential for the structure and function of antibodies. Antibodies do not naturally contain free sulfhydryl groups. It is hypothetically attractive to engineer an antibody to possess a cysteine the thiol group of which is neither oxidised nor compromises the fidelity of the natural sulfhydryl bridges.Sulfhydryl groups of cysteine residues may of course be exploited for conjugation by numerous conjugation chemistries that are rather specific for sulfhydryls such as maleimides, alkyl and aryl halides, α-haloacyls and pyridyl disulfides. However, it has been determined that variant monoclonal antibodies that have been designed with unnatural cysteine residues do not *de facto* possess free thiol groups available for conjugation. (Since antibodies consist of two identical heavy and two identical light chains and depending on how the gene has been engineered and expressed, genetic replacement of a residue on one chain can result in two new residues in the complete H2-L2 structure; furthermore, the two residues need not be chemically identical due to their spatial arrangement and neighbourhood.) In one known example of an engineered thiol (Bodmer, *et al.*, US # 5,219,996 and Lyons, *et al., Protein Eng. 3:* 703, 1990), an available free thiol was observed only when a cysteine residue was introduced into a discreet "concave" molecular pocket within the antibody structure -- a pocket only accessible to small molecules (0.13 - 0.5 mm diameter) and inaccessible for forming disulfide bridges within the same or other antibody chains. Cysteine residues introduced on "convex" or "flat" surfaces of the monoclonal antibody were found not to contain free thiols. One

skilled in the art would therefore conclude that surface cysteine residues would form disulfide bridges within the same and/or another antibody chain and thus distort the macromolecular structure and functions of the antibody.

The published literature indicates that engineered surface thiols alter antibody structure and function. For example, both monomers and dimers (IgG-IgG) were observed in the crude antibody-producing cell supernatant of a ser_{444}→cys variant (Shopes, *J. Immunol. 148:* 2918. 1992). Relatedly, a procedure was described for the production of dimeric antibodies also using a ser_{444}→cys variant (Caron, *et al., J. Exp. Med. 176*: 1191-1195, 1992). The dimeric IgGs from both of these examples were found to have enhanced effector functions *in vitro*. In these examples and in the earlier example of Bodmer, *et al.*, no physicochemical evidence was provided to indicate whether dimers formed specifically at the engineered site or to what extent normal disulfide bridges may have been altered. Finally, a "tethered" antibody has been described that was produced by creating a ser_{119}→cys variant (Shopes, *Mol. Immunol. 30:* 603, 1993). The variant allegedly generated dimers and an interchain structural variant or "tethered" antibody. Mixtures of structural aberrations compromise the goal of producing a defined chemical entity.

It was therefore with some surprise and contrary to expectation that we found that monoclonal antibodies containing a cysteine residue exposed on the surface of the antibody, are, in fact, amenable to site-directed conjugation, especially those containing a ser_{442}→cys heavy chain variant.

Accordingly, the present invention provides a monoclonal antibody comprising a cysteine residue exposed on the surface of the antibody such that the residue is capable of being conjugated to a substance and wherein the antibody is immunochemically functional, the term immunochemically functional primarily referring to the antibody's ability to bind but also encompassing effector functions if these are present. Bearing in mind that the variable region of the antibody contains the antigen binding site the preferred sites for conjugation are the surface residues on the surface of the variable region which are not involved in antigen binding such as, for example, the sSv heavy chain - light chain linker peptide as well as the surface residues of the constant region of the antibody which encompass the constant region of the light chain, the CH1,CH2, and CH3 domains of the heavy chain and also includes the hinge region. Thus all

residues on the surface of the antibody which are not involved in antigen binding, in particular those which are not part of the CDRs, are suitable for conjugation to a substance. If the antibody has effector functions, the preferred sites for conjugation are the same but excluding the CH2 domain which is known to contain the effector functions. The cysteine residue is in a substantially reduced form. More preferably, the reduced cysteine residue is in the CH3 domain of the heavy chain and more preferably at position 442 within the CH3 domain. Another preferred position for the reduced cysteine residue is the heavy chain - light chain linker peptide. Novel cys_{442} antibodies are capable of being expressed by their producer cells in a manner indicating both monomeric IgG and aggregated forms. Although the presence of aggregate suggested that the cys_{442} variants were surface variants as had been observed in the works by Bodmer, *et al.*, and by Shopes, surprisingly, most of the antibody was not in an aggregated form. Monomeric IgG was readily purified, for example, by gel filtration chromatography, and the monomeric form was stable upon long term storage. The monomeric IgG was found to possess no free thiol (Table 1). Whilst not wishing to be bound by theory, we believe that the thiol may be initially blocked (i.e. protected) by naturally occcurring adducts such as for example, glutathione.

We have discovered that the engineered thiol is reduced under controlled conditions that do not reduce the natural disulfide bonds. For example, milder conditions such as lower concentrations of the reductant which are not capable of reducing the natural disulfide bonds are found to be suitable for reducing the engineered thiol . The reduced antibody sustains a monomeric form even when stored for prolonged periods of time at pH 8. (Thiols are known to be reactive by judicious manipulation of pH and oxygen). Thus, by controlled reduction, the engineered antibody is rendered capable of site-directed chemical addition specifically at the engineered thiol.

Thus the present invention is also directed to a monoclonal antibody comprising a cysteine residue exposed on the surface of the antibody wherein, by controlled reduction, the antibody is rendered capable of site-directed chemical conjugation to a substance, said cysteine residue being introduced at a site which does not interfere with the immunochemical function of the antibody.

The antibodies according to the present invention are preferably monoclonal antibodies, or fragments thereof, the term antibody encompassing both antibodies and antibody

fragments. Antibodies according to the present invention can be from any species. The antibodies may be chimaeric antibodies that have variable regions from one antibody and constant regions from another, such as a human antibody. Thus, chimaeric antibodies may be species/species chimaeras or class/class chimaeras. Such chimaeric antibodies may have one or more further modifications to improve antigen binding ability or to alter effector functioning. Another form of altered antibody is a humanised antibody including a composite antibody, wherein the constant regions and the hypervariable regions other than the CDRs are transferred to the human framework. Additional amino acids in the framework or constant regions of such antibodies may be altered if required to restore binding. Thus the antibodies of use in the present invention include any altered antibodies in which the amino acid sequence is not one which exists in nature. However, CDR-grafted antibodies are most preferred. Antibodies of the present invention include different isotypes such, for example, as G1, G2, G4. Examples of antibodies are the 40KD antibody (CO/17.1.A) as disclosed in J. Cell Biol 125(2) 437-446, April 1994 and in Proc.Natl. Acad. Sci. 87, 3542-3546, May 1990, preferably the humanised anti-40KD antibody and in particular humanised anti-40KD of the G4 isotype. A specific example of an anti-40KD antibody is 323/A3, preferably humanised 323/A3 and in particular humanised 323/A3 IgG4.

Another example of an antibody is an anti-folate recepter antibody as disclosed in A.Tomasetti *et* al, Federation of European Biochemical Societies Vol 317, 143-146, Feb 1993, preferably humanised anti-folate and in particular humanised anti-folate of the G1 isotype. A specific example of an anti-folate antibody is MOV18, preferably humanised MOV18 IgG1. Further examples of antibodies include anti-CEA, anti mucin, anti-20/200KD, anti-ganglioside, anti-digoxin, anti-CD4, anti-CD23, anti-CDw52 and more specifically Campath-1H which is a humanised anti-CDw52 antibody. The antibody chain DNA sequences including the CDRs of Campath-1H™ are set out in EPO328404, the disclosure of which is hereby incorporated by reference. (Page, M.J., and Sydenham, M.A., High level expression of the humanised monoclonal antibody Campath-1H in Chinese Hamster Ovary cells, Biotech. 9: 64-68, 1991.).

Antibody fragments of use in the present invention include Fab, F(ab)2, Fv and fragments comprising synthetic peptide sequences eg. as generated by recombinant DNA technology.

Monoclonal antibodies of use in the invention may be prepared by any method well known in the art or more particularly as described in GB 9022547.5. Purification may be carried out as described in EP-A-91917891.

Fragments may be prepared by any of the means known in the literature. for example Antibodies, a laboratory manual, eds. E. Harlow and D. Lane, Cold Spring Harbor Laboratory, 1988 or by molecular genetic means.

The invention also provides an antibody wherein the cysteine is conjugated either directly or indirectly to a substance. When the substance is conjugated indirectly to the antibody it may be connected to the antibody via one or more linkage moieties such as for example chelators. Such substances which are connected to the antibody via one or more linkage moieties are commonly known as "bifunctional substances". Such linkage moieties may, for example, be a functional chemical moiety such as maleimide or bromoacetyl, that is capable of covalent attachment to thiol functional groups within proteins such as antibodies. The linkage moiety may also utilise a chemical spacer (e.g. a p-benzyl group) that functions as a bridge between the substance(s) and the antibody attachment moiety.

Examples of the conjugated substance are dyes, radionuclides, enzymes, drugs, cytotoxins, and biotin/avidin. Specific examples of a drug and cytotoxin being adriamycin and ricin respectively. Specific examples of chelators include the following.

DOTA (1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid);
PA-DOTA(α-[2-(*p*-nitrophenyl)ethyl]-1,4,7,10-1-acetic-4,7,10-tris(methylacetic)acid);
TMT (6,6"-bis[*N,N'',N'''*-tetra(carboxymethyl)aminomethyl)-4'-(3-amino-4-methoxyphenyl)-2,2':6',2"-terpyridine);
IB4M-DTPA (*N,N,N',N'',N''*-pentakis(carboxymethyl)-2-[(4-aminophenyl)methyl]-6-methyldiethylenetriamine);
CHX-A-DTPA (N-[2-amino-3-(*p*-aminobenzyl)propyl]-*trans*-cyclohexane-1,2-diamine-*N,N',N',N'',N''*-pentaacetic acid);
TRITA (1,4,7,10-tetraazacyclotridecane-*N,N',N'',N'''*-tetraacetic acid); and
TETA 1,4,8,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid

When the substance is a radionuclide, it may be attached to the free thiol of the antibody by either direct or indirect methods. For example, ^{99m}Tc may be directly attached to the antibody by a modification of procedures similar to the Schwartz method (Schwartz, A., and Steinstrasser, A., J. Nucl. Med. 18:721, 1987), wherein reduction of the natural disulfides would not be necessary. Metallic radionuclides such as ^{90}Y, ^{186}Re, ^{177}Lu, ^{111}In and ^{67}Cu may be attached by either indirect prelabelling methods, wherein radionuclide is first added to bifunctional chelator then conjugated to antibody, or by indirect postlabelling methods, wherein radionuclide is added to preformed chelator-antibody conjugate. Chelators of the present invention may be linked to antibodies by any hetero- or homo-bifunctional cross linker (i.e. chemical spacer) capable of linking a chelator to a thiol group in the antibody (M.McCall et al., Bioconjugate Chem. 1, 222-226, 1990) or by use of cross linkers described in Pierce "Immuno Technology Catalog and Handbook" 1992, pages E8 to E39 and in Parker, Chem.Soc.Rev. [1990], 19, 271-291 and the methods referred to therein.

Examples of chelators in combination with cross-linkers include:

bromoacetyl-DOTA (2-[*p*-(bromoacetamido)benzyl-1,4,7,10-tetraazacyclo-dodecane-*N,N',N'',N'''*-tetraacetic acid);
bromacetyl-TRITA (2-[*p*-(bromoacetamido)benzyl]-1,4,7,10-tetraazacyclotridecane-*N,N',N'',N'''*-tetraacetic acid);
bromoacetyl-TMT (2-[*p*-(bromoacetamido)benzyl]-6,6''-bis[*N,N'',N''*-tetra(carboxymethyl)aminomethyl)-4'-(3-amino-4'-methoxyphenyl)-2,2' : 6',2''-terpyridine);

The bifunctional chelator cross-linker combination, bromacetyl-DOTA, also called BAD, is described in M.J. McCall, H. Diril, and C.F. Meares, *Bioconjugate Chem. 1:* 222-226, 1990

Most particularly the chelator of use in the present invention is either DOTA or TMT and the protein reactive group (cross-linker) is either bromoacetyl or a maleimide, most preferably bromoacetyl.

Chelators of use in the present invention may be prepared by any known technique (for example M.McCall et al., ibid)

Radionuclides which may be used in accordance with the present invention include those appropriate for obtaining in vivo radioimmunotherapy and/or imaging of a target cell or tissue. For radioimmunotherapy, a high dose of energy must be delivered to the target site in order that cellular DNA is damaged; both α and β emitting radionuclides produce emissions in a suitable energy range. However α-emitters are either shorter lived or decay to hazardous daughter products. Hence the radionuclide of choice for radioimmunotherapy will usually be a β-emitter. For imaging the radiation must interact as little as possible with the body tissue yet produce a strong signal for external detection. Hence a gamma emitting radionuclide is most suitable for imaging. For both imaging and radioimmunotherapy the radionuclide must possess a half-life suitable to permit activity or detection after the elapsed time between administration and binding to the target site. The radiolabelled antibody must travel from the bloodstream to the extracellular fluids of the target via the endothelial pores. Large antibodies or antibody/chelator complexes may diffuse slowly and a radionuclide half-life of between several hours and several days is desirable. In a particular aspect of the present invention the radionuclide is selected from the group comprising of ^{195m}Pt, ^{57}Ni, ^{57}Co, ^{105}Ag, ^{68}Cu, ^{52}Mn, ^{52}Fe, ^{111}In, ^{113m}In, ^{99m}Tc, ^{67}Ga, ^{169}Yb, ^{166}Tm, ^{167}Tm, ^{146}Gd, ^{157}Dy, ^{95m}Nb, ^{103}Ru, ^{97}Ru, ^{99}Ru, ^{101m}Rh, ^{201}Tl, ^{203}Hg, ^{197}Hg, ^{203}Pb, ^{99}Rh, ^{48}Cr, ^{57}Co, ^{125}I, ^{131}I, ^{35}S, ^{153}Sm, ^{88}Y, ^{90}Y, ^{186}Re, ^{188}Re, ^{211}At, ^{212}Bi, ^{212}Pb and ^{177}Lu.

In a more particular embodiment of the present invention the radionuclide is selected from the group comprising ^{111}In, ^{67}Cu, ^{186}Re, ^{188}Re, ^{177}Lu, ^{99m}Tc, ^{131}I, ^{88}Y, ^{90}Y, ^{211}At, ^{212}Bi ^{212}Pb ^{57}Co, ^{153}Sm., ^{88}Y ^{90}Y and ^{177}Lu.

In a more particular embodiment of the present invention the radionuclide is ^{177}Lu ^{153}Sm ^{90}Y and ^{111}In. The invention also provides a radiolabelled antibody comprising an antibody of the present invention conjugated either directly or indirectly to a radionuclide, in particular chelator-antibody conjugate that may be labelled with ^{90}Y or ^{177}Lu via DOTA, or TMT. The invention also provides methods for producing antibodies capable of being conjugated at specific sites and for site-directed conjugation of antibodies according to the invention.

According to another aspect of the present invention there is provided the use of a conjugated antibody of the invention in therapy and diagnosis. In particular there is

provided the use of antibodies according to the invention for the diagnosis and/or therapy of conditions which are detectable or amenable to therapy with dyes, radionuclides enzymes, drugs and cytotoxins. These antibody complexes are useful in treating cancers such as lymphomas and leukaemias and in particular small cell and non small cell lung cancer, prostatic cancer as well as ovarian cancer.. In a most particular aspect of the present invention there is provided an antibody complex according to the invention for use in the imaging and/or treatment of cancers and associated metastases. They may also be used for example as immunosuppressives and more particularly for the treatment of T-cell mediated disorders including severe vasculitis, rheumatoid arthritis, systemic lupis, also autoimmune disorders such as multiple sclerosis, graft vs host disease, psoriasis, juvenile onset diabetes, Sjogrens' disease, thyroid disease, myasthenia gravis, transplant rejection and asthma.

The invention also provides the use of a conjugated antibody described above in the manufacture of a medicament for the treatment or imaging of any of the aformentioned disorders.

According to another aspect of the present invention there is provided a method of treatment of conditions amenable to therapy and diagnosis with a conjugated antibody complex according to the invention comprising administering a therapeutically efficacious amount of antibody complex to a mammal requiring such treatment. In particular there are provided methods of treatment of cancers such as lymphomas and leukaemias and in particular small cell and non-small cell lung cancer, prostatic cancer as well as ovarian cancer and most particularly methods of treatment of cancers and associated metastases. They may also be used in a method of treatment of T-cell mediated disorders including severe vasculitis, rheumatoid arthritis, systemic lupis and also autoimmune disorders such as multiple sclerosis, graft vs host disease, psoriasis, juvenile onset diabetes, Sjogren's disease, thyroid disease, myasthenia gravis, transplant rejection and asthma.

There is also provided in the present invention a pharmaceutically acceptable composition containing conjugated antibodies according to the present invention which

comprise a conjugated monoclonal antibody or fragment thereof and one or more pharmaceutically acceptable excipients.

Such compositions include, in addition to conjugated antibodies a physiologically acceptable diluent or carrier possibly in admixture with other agents such as other antibodies or an antibiotic. Suitable carriers include but are not limited to physiological saline, phosphate buffered saline, phosphate buffered saline glucose and buffered saline. Routes of administration are routinely parenteral including intravenous, intramuscular, subcutaneous and intraperitoneal injection or delivery.

In respect of radioimmunotherapy the dosages of compositions containing antibody conjugated to radionuclides according to the invention will vary with the condition being treated and the recipient of the treatment, but will be in the range of to about 1-100mg for an adult patient, preferably 1-10mg, most preferably 5mg, usually administered as an infusion. A repeat dosing regime may be preferable wherein 10 mg are administered for 1 day followed after weeks or months by a second treatment.

In respect of imaging the dosages of such compositions will vary with the condition being imaged and the recipient of the treatment, but will be in the range 1 to about 100mg, preferably 1-10 and most preferably 5mg for an adult patient.

Kits can also be supplied for use with the subject conjugated antibodies in the protection against or detection of a cellular activity or for the presence of a selected antigen. Thus, a monoclonal antibody conjugated of the present invention may be provided, usually in a lyophilized form in a container, either alone or in conjunction with additional antibodies specific for the desired cell type. The conjugated antibodies, which may be conjugated to a dye, radionuclide, enzyme, drug, cytotoxin, chelator or biotin/avidin, are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilisers, biocides, inert proteins, e.g., serum albumin, or the like, and a set of instructions for use. Generally, these materials will be present in less than about 5% wt. based on the amount of active antibody, and usually present in total amount of at least about 0.001% wt. based again on the antibody concentration. Frequently, it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1 to 99% wt. of the total composition. Where a second antibody capable of binding to the chimeric antibody is employed in an assay, this will usually be

present in a separate vial. The second antibody is typically conjugated to a label and formulated in an analogous manner with the antibody formulations described above. Generally the kit will also contain a set of instructions for use.

Description of Figures

Figure 1. Immunoreactivity of variant antibodies.

a) Five distinct antibody preparations bearing the Campath-1H antigen specificity -- 2 natural isotypes (G1 and G4) and 3 variants (G4m, G4c and G4mc), the G4c and G4mc containing ser $_{442}\rightarrow$ cys substitutions -- were constructed and expressed in NSO cells as described in example 1. The antibodies were labelled in situ with ^{35}S and purified in the following manner. Two x 10^7 washed producer cells were incubated for 48-72hrs at 37° in 5ml methionine cystine-free DMEM (ICN Biomedicals, Costa Mesa, CA) that contained 6µg cystine/ml, 3µg methionine/ml, and 8-10mCi of ^{35}S methionine eg. Tran ^{35}S-label (ICN). The supernatant was harvested by centrifugation, dialized and concentrated (Centricon, Amicon, Beverly, MA). The antibody was purified by protein A chromatography (HPLC Dynamax Hydropore-protein A mini column [Rainin, Woburn, MA]), eluted with 1 M acetic acid, concentrated by ultrafiltration eg. verus a PM 30 membrane (Amicon) and further fractionated by HPLC gel filtration eg. S-5 200A diol, YMC, Willmington, NC. The protein A eluants resolved on gel filtration commensurate with unlabelled antibodies and had specific activites ~ 2µCi/µg. Immunoreactivity was determined on Fixed Wein 133 C1 cells using the method of Lindmo *et al.* (*J. Immunol. Meth. 72:* 77-84, 1984). Note that the *y* intercept, the inverse of which is a measure of immunoreactivity, was equivalent for all the preparations. (The slopes are a function of the specific activity and not the immunoreactivity.) b) Equilibrium specific binding competition for Campath-1H G1 and G4mc on fixed Wein 133 C1 cells. The two antibodies were biosynthetically labelled in situ with ^{35}S to the same specific activity (0.3 mCi/nmole) and purified (^{35}S-ligand) as described above. Equilibrium competition was carried out in parallel where each ligand was competed for by its respective unlabelled form. Binding was performed in a total volume of 0.2ml containing 5 x 10^4 fixed Wein 133 cells, ~0.1 nM of the indicated radiolabeled form of C1H and the indicated concentration of competitor. The binding buffer contained phosphate-buffered saline, 2% bovine calf serum, 0.01% triton X100, and 0.02% sodium azide (binding buffer) overnight at 4°. The cells were

centrifuged, washed 3 times with cold binding buffer and radioactivity determined. Points depict the average of triplicate measurements ± SEM (bars). The two antibodies were biosynthetically labelled in situ with ^{35}S to the same specific activity (0.3 mCi/nmole) and purified (^{35}S-ligand) as described above. Equilibrium competition was carried out in parallel where each ligand was competed for by its respective unlabelled form. The two profiles indicate that the antibodies have identical antigen binding potencies.

More definitive evidence for site-directed conjugation was obtained by radiolabeling a monoclonal antibody chelator conjugate prepared as described in Example 3. Peptide mapping (enzymatic digestion, fractionation of peptides and peptide amino acid sequence analysis) determined that all radioactive peaks were composed of heavy chain C-terminal peptide fragments that contained cys442 -chelator adduct.

Figure 2. Site specific conjugation as indicated by SDS PAGE.

A $ser_{442}\rightarrow$ cys variant, Campath-1H G4mc, was specifically reduced as described in Example 2. By way of comparison, thiol groups were introduced onto nonreduced antibody using the procedure of McCall, *et al., Bioconjugate Chem. 1:* 222-226, 1990. (The latter procedure is a random conjugation process that employs 2-iminothiolane to introduce thiol groups onto the ε amino groups of lysine residues.) Both thiol-containing antibody preparations were then conjugated as described in Example 2. Equal amounts of the labelled conjugates were subjected to reducing SDS PAGE, wherein the antibody heavy and light chain subunits were separated by virtue of their size. The gel was stained for protein (left side) and for radioactivity by autoradiography (right side). Lanes, left to right: Random, protein stain; specific, protein stain; random, autorad; specific, autorad. The figure shows that the addition of the conjugate was localised to the heavy chain for the specific labelling procedure, whereas for the random process, both subunits were labelled.

Figure 3.

Biodistribution of ^{90}Y-TMT-Campath-1H G4mc conjugate. Reduced Campath-1H G4mc was conjugated to bromoacetyl-TMT, radiolabeled with $^{90}YCl_3$ and biodistribution carried out in tumor-bearing mice as described above for bromoacetyl-

DOTA. bars show the average % injected dose/g tissue (% ID/g) corrected for decay for 5 mice.

The following Examples are illustrative of the present invention and not intended to constitute any limitation thereof:

Example 1. production of variant monoclonal antibodies with a cys substitution.

(a) Campath-1H and anti-digoxin variants. Cys_{442} was introduced into the heavy chain of various isotypes by conventional molecular genetic means. For example, genetic constructs of human IgG2, IgG4, Campath-1H and anti-digoxin (both IgG1) were obtained from within Wellcome Laboratories. The terminal portion of the CH3 region was excised with the restriction enzymes NsiI(5') and EcoR1(3') and replaced with an annealed double stranded oligonucleotide ligated at the respective restriction sites. Antigen specificity was introduced onto constant regions by respective replacement of the variable and CH1 regions. A $ser_{228}\rightarrow pro$ was introduced into IgG4 in order to match the G4 sequence initially reported by Pink, *et al., Biochem. J. 117:* 33-47, 1970. An IgG4 cys_{442} variant entitled G4mc was further modified by changing three residues in the CH2 region: $leu_{235}\rightarrow ala$, $gly_{237}\rightarrow ala$ and $glu_{318}\rightarrow ala$. these latter changes were introduced based on rationale supplied by Winter, *et al.*, that such changes might reduce antibody interaction with Fc gamma receptors and complement C1q (Duncan, *et al., Nature 332:* 563-564, 1988; Duncan and Winter, *Nature 332:* 738-740, 1988).

(b) 323/A3 variant. The murine antibody 323/A3 reacts with an epitope on human epithelial tissues that may be useful in the identification of treatment of adenocarcinoma (Edwards, *et al.*, Cancer Res. 46: 1306-1317, 1986). The complimentarity determining regions within the variable region of 323/A3 were first "humanized" and grafted onto a human IgGI isotype. To prepare a cys variant, the cDNA expression construct was ligated in frame with the cDNA encoding the constant region of Campath-1H IgG4 cys442 variant. The humanized 323/A3 IgG4 cys442 variant was expressed in NSO cells and purified by conventional means.

(c) 323/A3 sFv fragment. A single chain sFv fragment of humanized 323/A3 was constructed by conventional PCR and cloning techniques. Cys variant constructs were

produced by introducing a cys residue substitutions into the linker region. For example, the conventional linker region (gly4ser three repeat) was altered to contain gly4sergly2cys2sergly4ser by site-directed mutagenesis. The variants were expressed in E. coli and purified by affinity chromatography.

(d) Mov-18 variant. Mov-18 reacts with a folate binding protein that is prominently expressed on ovarian cancer tissue (Miotti, *et al.*, Intl. J. Cancer 39: 297-303, 1987). A human IgG1 isotype cDNA was cloned from a public source mRNA library by using reverse transcriptase. The variable region of Mov-18 was humanized and ligated to the human G1 constant region. Cys442 was introduced into the heavy chain cDNA by site-directed mutagenesis. The humanized Mov-18 IgG1 cys442 variant was expressed in NSO cells and purified by conventional means.

Example 2. Reduction of variant monoclonal antibodies.

Antibody solutions were prepared in degassed buffer, such as 100mM sodium phosphate or trimethylammonium phosphate at pH> 8.0, preferably pH 8.0 - 8.5, at a convenient concentration, for example 100 μM (15 mg/ml). An amount of reductant was mixed with the antibody solution to achieve the desired extent of reduction. For example, fixed volumes of a 50% gel slurry of a solid phase reductant such as Reduce-Imm^{TM} (Pierce) were stirred into the antibody solution. The reductant capacity of Reduce-Imm^{TM} gel was assumed from the manufacturer to be 30 μmole/ml packed gel. A 250 μl volume of 50% slurry was added to 1 ml of 100 μM antibody solution to generate a mixture with a 30-fold excess reductant capacity (mole reductant/mole antibody). The antibody-reductant mixture was shaken for 1 hr at room temperature, centrifuged and the solution subjected to additional procedures such as free thiol determination, pH reduction, purification or conjugation as described in Example 3.

Alternatively, antibody protein was reduced with soluble reductant such as mercaptoethylamine. For example, protein was concentrated to 200-300 μM in 0.1 M sodium phosphate, pH 6.0, 5 mM DTPA. Mercaptoethylamine was added to a final concentration 10-fold in excess of the protein concentration and mixed gently for 1 hour at room temperature. The reduced protein was then separated from reductant and prepared for conjugation by conventional means such as gel filtration. Commonly, protein solution was gel filtered through Bio Spin 30 columns (Bio-Rad Laboratories)

that had been pre-equilibrated in 0.1 M tetramethylammonium phosphate pH 8.2, 25 uM DTPA for 2 min at 150 x g.

Example 3. Site directed conjugation of reduced variant antibody.

A reduced $ser_{442}\rightarrow$ cys variant, Campath-1H G4mc, was conjugated to 2-[p-(bromoacetamido)benzyl]-1,4,7,10-tetraazacyclododecane-*N,N'N'',N'''*-tetraacetic acid (bromoacetyl-DOTA). Bromoacetyl-DOTA was a bifunctional moiety wherein one aspect was a chelator that had been prelabelled with ^{57}Co and the second aspect was a reactive group capable of covalent attachment to a free sulfhydryl. The bifunctional chelator and a method for labelling it with ^{57}Co has been described previously (Mears, *et al., Analytical Biochem. 142:* 68-78, 1984;). Briefly, a solution of bromoacetyl DOTA was trace labelled with ^{57}Co, and then a 10-fold molar excess was added to antibody solution. The conjugation reaction was carried out for 2 hrs at 37° and stopped by separation of the reactants by gel filtration using a Bio Spin 30 column (Bio Rad) as recommended by the manufacturer. Bifunctional chelators, such as bromoacetylTMT, were also employed prior to radiolabeling. The termperature and duration of the conjugation reaction was varied for maximal site-specific conjugation. For example, reduced protein at a concentration of 150 - 200 uM in tetramethylammonium phosphate, pH 8.2, 25 μM DTPA, was added to a 10-fold excess of bromoacetylTMT in a metal-free reaction vial. The reaction was carried out for 24 hours at room termperature, and the conjugate was isolated by gel filtration as described above.

Example 4. Murine biodistribution of conjugates.

A reduced $ser_{442}\rightarrow$ cys variant, Campath-1H G4mc, was conjugated to bromoacetyl DOTA by the site directed procedure described above without prelabelling. By way of comparison, random conjugate was prepared by introducing thiol groups into nonreduced Campath-1H g4mc as has been described previously for Lym-1. (The latter process follows the procedure of McCall, *et al., Bioconjugate Chem. 1:* 222-226, 1990 and employs 2-iminothiolane to introduce thiol groups randomly onto the ε amino groups of lysine residues.) Iminothiolated Campath-1H G4mc was conjugated to bromoacetyl-DOTA to the same extent (chelators per antibody) as site directed conjugate. The conjugates were rendered radioactive by mixing with $^{90}YCl_3$ in plasticware by addition of the following reagents: an 8X volume of monoclonal antibody conjugate (> 25 mg/mL, 0.1 M ammonium acetate pH 6.7), a 2X volume of ^{90}Y, and cold yttrium up to a final concentration of 10 μM. Cold yttrium was added

first. followed by ^{90}Y and conjugate. Chelation was allowed to occur at room temperature for 90 minutes. Ten μg (2 μCi) were injected intravenously per mouse into mice bearing a subcutaneous tumour (CHO-10/D4) that expressed the Campath-1H antigen. Mice were sacrificed at the indicated times. tissues excised. weighed and radioactivity determined. The results are shown in Table II below and are expressed as the % injected dose per gram tissue (average of 5 mice per group +/- sem). Note the diminution of normal tissue deposition and increase in tumour deposition in the site-directed (direct) conjugate groups relative to the random groups. Figure 3 illustrates the biodistribution of ^{90}Y-TMT-Campath-1HG4mc.

Example 5

A reduced ser_{442}→cys variant. Campath-1H was conjugated to TMT, that is covalently attached via a thioether linkage to the cys442 residues in the heavy chains as illustrated in Figure 4. The reaction may be regulated to produce conjugates that contain an average of 1 - 2 chelators per antibody. Conjugate is purified free of unreacted bifunctional chelator by gel filtration in metal-free conditions and is stable in a buffered, metal-free environment.

Immunoconjugate chelation. Conjugates were radiolabeled in metal-free plasticware using the best metal-free reagents available. Carrier-free $^{90}YCl_3$ was purchased from Dupont/New England Nuclear. Amersham and other sources. The specific activity was typically 5 mCi in 10-30 μL 0.05 N HCl, specific activity 5.6 X 10^5 Ci/g. Prior to use, the $^{90}YCl_3$ was buffered with 0.1 volume of 6 M ammonium acetate to ~ pH 5.8. Chelation was performed by adding the following reagents in sequence and incubating for up to 90 min at room temperature: a 1X volume of cold yttrium (100μM yttrium in 0.1 M ammonium acetate, pH 6.8), a 2X volume of ^{90}Y acetate. and an 8X volume of monoclonal antibody conjugate (25 mg/ml 0.1 M ammonium acetate, pH 6.5). Non-chelated radiometal was "scavenged" by the addition of DTPA to a final concentration of 500μM and a 10X volume of 0.1 M ammonium citrate, pH 6.5. The mixture was incubated at room temperature for 30 minutes and fractionated by "spin column gel filtration," i.e., applied to a 1 mL Bio-Spin 30 (Bio-Rad Laboratories) that had been pre-equilibrated in phosphate-buffered saline and centrifuged at 150 x g for 2 minutes. Spin column gel filtration was repeated for a total of two centrifugations.

The efficiency of chelation (ability to chelate all the radiometal) and scavenging (ability to remove non-chelated radioactivity from radiolabeled conjugate) was monitored by thin layer chromatography as described by Meares *et al.*, *Anal. Biochem. 142*, 68-78. 1984. More than 90% of the radiometal was routinely chelated. (The extent of chelation was dependent on the acid and metal content of the supplied radiometal.) After scavenging, the fraction of ^{90}Y that was tightly bound to conjugate was typically >98% when analyzed by HPLC gel filtration, thin layer chromatography or SDS PAGE.

^{90}Y-postlabeled conjugates have been prepared with specific activities of up to 10 mCi/mg which is 30-fold below the theoretical capacity. Although every chelator is available to accept radiometal, higher specific activity causes radiolysis (a function of time and concentration) that can be reduced by inclusion of an anti-oxidant such as ascorbic acid. In principle, chelation can be optimized for complete efficiency given a consistent and high quality supply of radiometal and thus eliminate the need for scavenging.

Example 6. Large scale reduction of variant monclonal antibodies

Dilute ultrafiltered IgG product to 50mg/ml (+/- 1mg/ml) and measure a volume V. Add V/10 of freshly prepared stock solution (13mg/ml MEA in 50mM phosphate and 5mM EDTA at pH7.0), mixing well during addition to ensure even distribution of reductant. Leave at ambient temperature for 60 minutes. The sample is applied to a gel filtration column such as, for example, Sephadex G25 or Superdex 75 or 30. The IgG peak is monitored at absorbance of 280nm and the peak collected and subjected to additional procedures such as free thiol determination, pH adjustment, purification or conjugation as described in Example 3.

Example 7. Large scale site directed conjugation of reduced variant antibody.

Add 10 fold molar excess of bromoacetyl-TMT as a 10mM stock made up in 0.1M Hepes + 25μM DTPA at pH8.4 over the IgG from example 6. Mix well and leave at ambient temperature for a minimum of 21 hours, maximum 37 hours. The conjugated antibody is then loaded onto a Superdex 200 column. The IgG peak is monitored at absorbance of 280nm and the monomer peak collected and subjected to standard analytical procedures such as estimation of binding, protein content etc.

Table 1.

Free thiol content following exposure to solid-phase reductant

A G4 $ser_{442}\rightarrow$ cys variant labelled "G4mc" and a natural G4 control were exposed to solid phase reductant as described in Example 2. The thiol content was determined by Ellman's reagent and is expressed relative to moles antibody. (It is assumed that 2 moles of thiol were reduced per antibody thiol.) The sem for triplicate measurements was $\pm$ 0.1 SH/antibody.

Molar Ex Gel	Moles -SH/Antibody	
	G4mc	G4
Expt. 1		
100	4.6	.6
30	3.0	.2
10	1.2	.1
3	.6	0
0	.1	0
Expt. 2		
50	3.3	.3
30	2.3	.2
20	1.7	.2
10	1.0	.1
3	.5	.1
0	.1	.1

Table II

Murine biodistribution of ^{90}Y-conjugates

% ID/g (avg -/- sem)

	24 hour		72 hour		168 hour	
direct						
BLOOD	13.47	1.75	8.80	1.67	5.93	0.80
SPLEEN	3.51	0.63	2.46	0.50	2.83	0.39
TUMOUR	17.01	3.16	24.78	8.41	31.36	4.96
LIVER	5.24	0.62	3.32	0.69	2.56	0.28
LUNG	5.60	0.49	5.00	0.70	3.99	1.32
KIDNEY	5.09	0.30	5.21	0.65	4.16	0.54
BONE	1.56	0.19	1.09	0.18	0.95	0.15
random						
BLOOD	11.97	3.96	9.48	2.00	6.66	0.76
SPLEEN	5.79	1.42	4.61	0.90	3.55	2.07
TUMOUR	15.42	5.52	22.37	9.29	29.91	4.70
LIVER	10.07	2.99	6.75	1.31	4.34	0.82
LUNG	6.19	0.95	4.56	1.22	3.67	1.08
KIDNEY	5.38	1.41	6.84	1.41	5.18	0.50
BONE	1.76	0.46	1.39	0.35	1.25	0.14

CLAIMS

1. An immunochemically functional monoclonal antibody comprising a cysteine residue exposed on the surface of the antibody such that the residue is capable of being conjugated to a substance.

2. An antibody according to claim 1, wherein the cysteine residue is in the variable region of the antibody but not part of the CDRs.

3. An antibody according to claim 1, wherein the cysteine residue is in the constant region .

4. An antibody according to claim 3, wherein the cysteine residue is in the CH3 domain of the heavy chain.

5. An antibody according to claim 4, wherein the cysteine residue is at position 442 within the CH3 domain.

6. An antibody according to any of the preceding claims, wherein the antibody binds to a 40KD antigen or folate receptor antigen.

7. An antibody according to any of the preceding claims, wherein the antibody is humanised.

8. An antibody according to any of the preceding claims, wherein the antibody is a G1 or G4 isotype.

9. An antibody conjugate comprising an antibody according to any of the preceding claims and a substance, which is directly or indirectly conjugated to the cysteine-residue of the antibody.

10. An antibody conjugate according to claim 9, wherein the substance is indirectly conjugated via a chelator.

11. An antibody conjugate according to claim 9, wherein the substance is indirectly conjugated via a linker and a chelator.

12. An antibody conjugate according to claim 10 or 11, wherein the chelator is TMT or DOTA.

13. An antibody conjugate according to claim 11 or 12, wherein the linker is bromoacetyl.

14. An antibody conjugate according to any of claims 10 to 13, wherein the substance is ^{90}Y or ^{177}Lu.

15. An antibody conjugate according to claim 9, wherein the substance is a molecular chimaera for use in enzyme-prodrug therapy.

16. An antibody conjugate according to claim 15, wherein the chimaera comprises a transcriptional regulatory DNA sequence capable of being activated in a mammalian cell and a DNA sequence operatively linked to the transcriptional regulatory DNA sequence and encoding a heterologous enzyme capable of catalysing the conversion of the prodrug into an agent toxic to the cancer cell.

17. An antibody conjugate according to claim 16, wherein the transcriptional regulatory DNA sequence is a tissue- or cancer- specific transcriptional regulatory DNA sequence.

18. An antibody conjugate according to claim 15 or 17, wherein the chimera is contained within a viral vector or liposome.

19. Use of an antibody conjugate according to any of claims 9 to 18, for the treatment and diagnosis of cancers and associated metastasis.

20. Use of an antibody conjugate according to claims 9 to 14, for the treatment of small cell and non-small cell lung cancer, prostatic cancer and associated metastasis.

21. Use of an antibody conjugate according to claims 15 to 18, for the treatment of ovarian cancer.

22. Use of an antibody conjugate according to claims10 to 14 for radioimmunotherapy.

23. A pharmaceutically acceptable composition comprising conjugated antibodies according to any of claims 9 to 18 together with a physiologically acceptable diluent or carrier.

24. A pharmaceutically acceptable composition according to claim 23 for radioimmunotherapy, wherein the dosage is 1 to 10 mg.

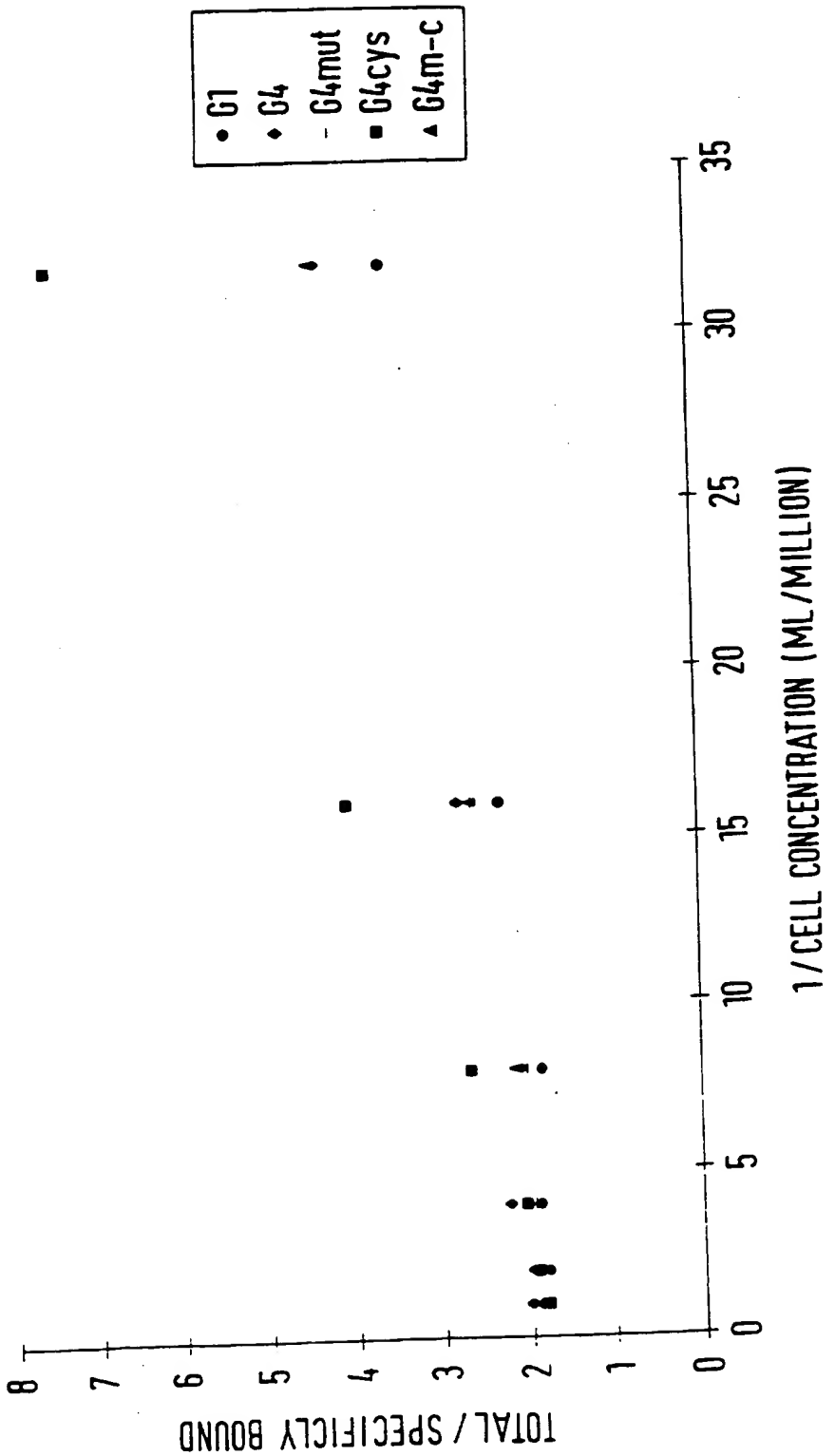
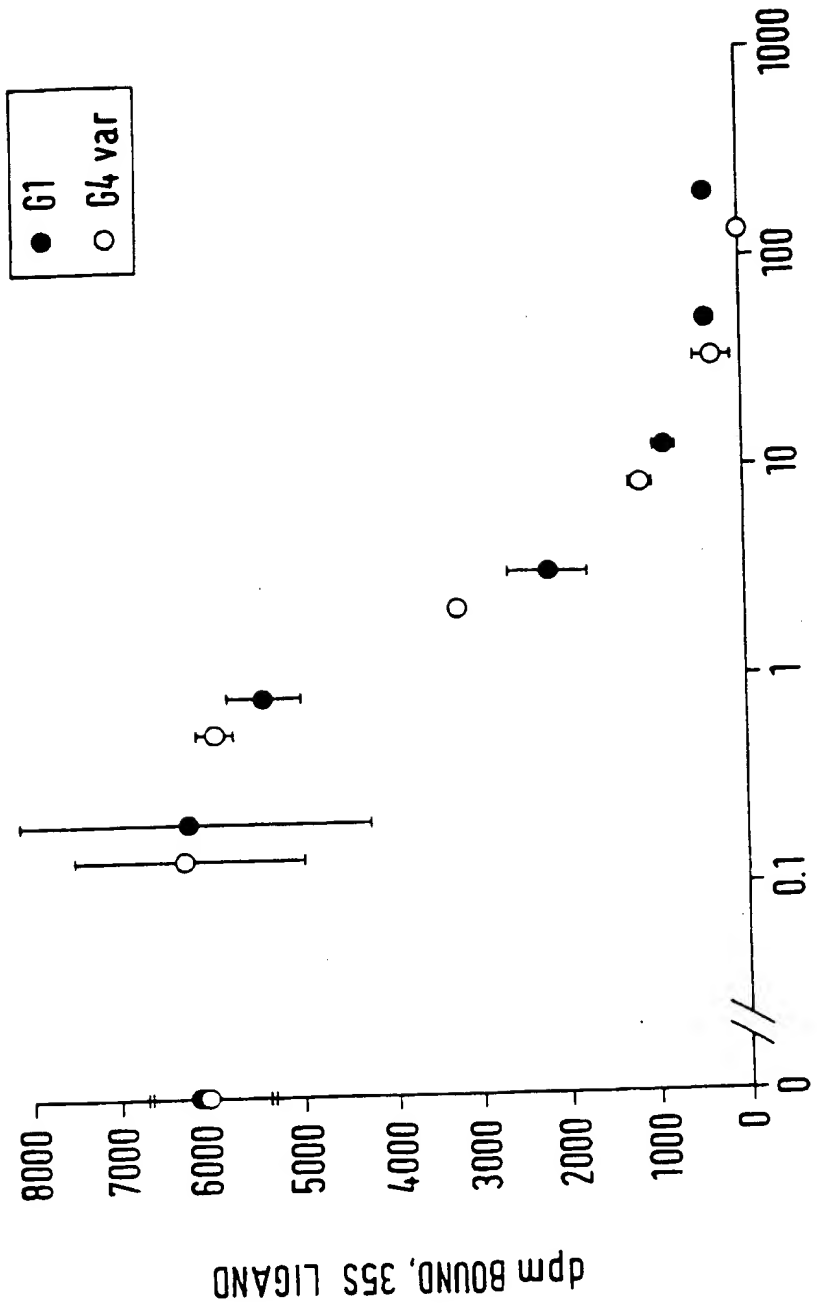

G1
G4
G4mut
G4cys
G4m-c
TOTAL / SPECIFICLY BOUND
0
1
2
3
4
5
6
7
8
1/CELL CONCENTRATION (ML/MILLION)
0
5
10
15
20
25
30
35

Fig. 1a


G1
G4 var
dpm BOUND, 35S LIGAND
8000
7000
6000
5000
4000
3000
2000
1000
0
0
0.1
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100
1000
nM UNLABELLD COMPETITOR

Fig. 1b

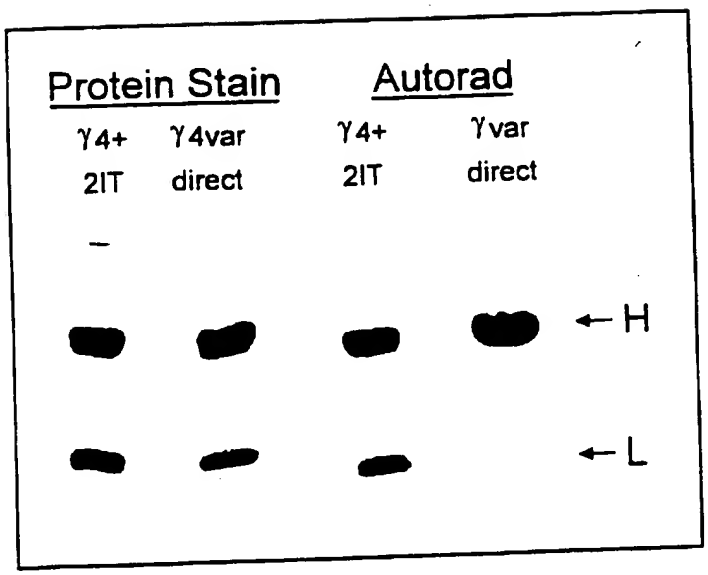
Protein Stain
Autorad
γ4+ 2IT
γ4var direct
γ4+ 2IT
γvar direct
←H
←L

Fig. 2

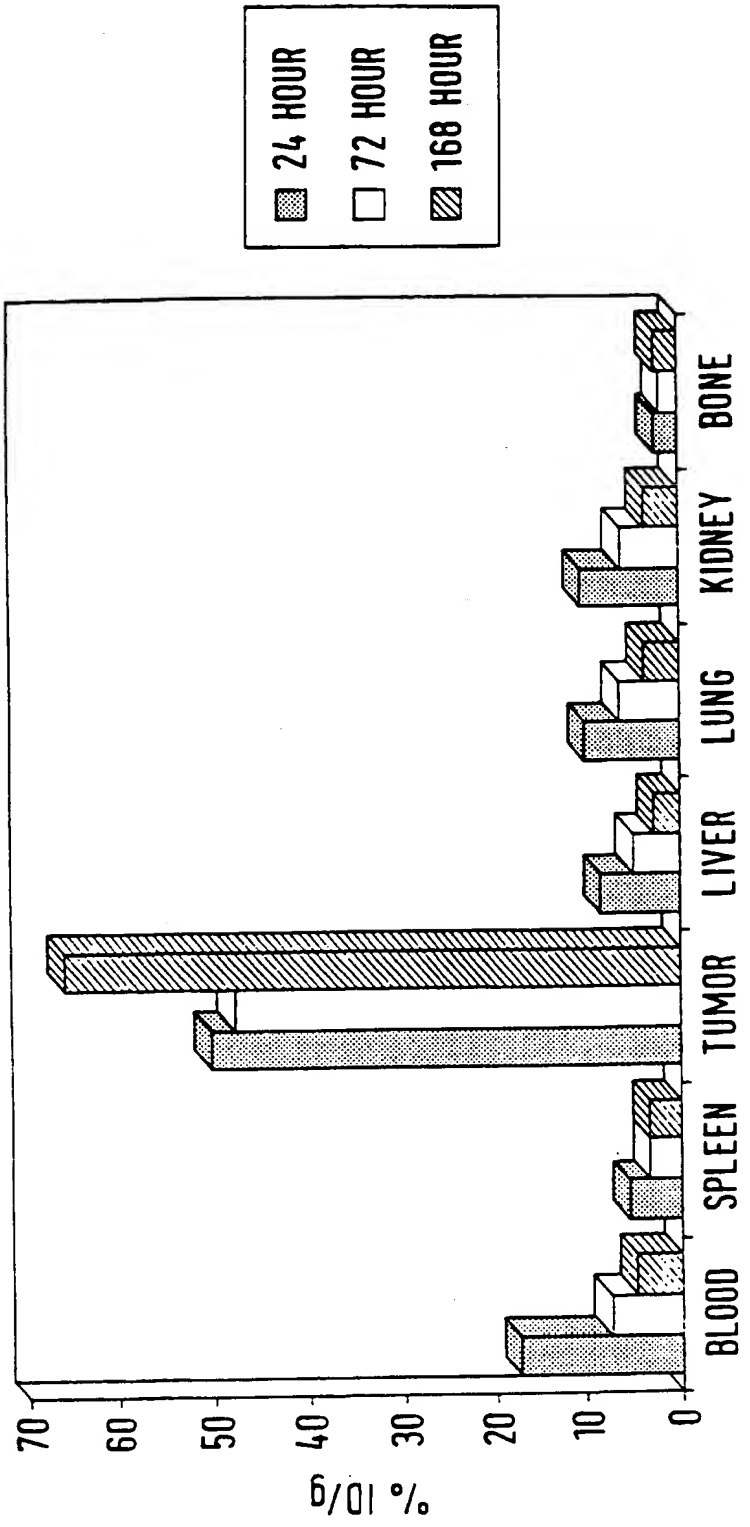
24 HOUR
72 HOUR
168 HOUR
% ID/g
0
10
20
30
40
50
60
70
BLOOD
SPLEEN
TUMOR
LIVER
LUNG
KIDNEY
BONE

Fig. 3

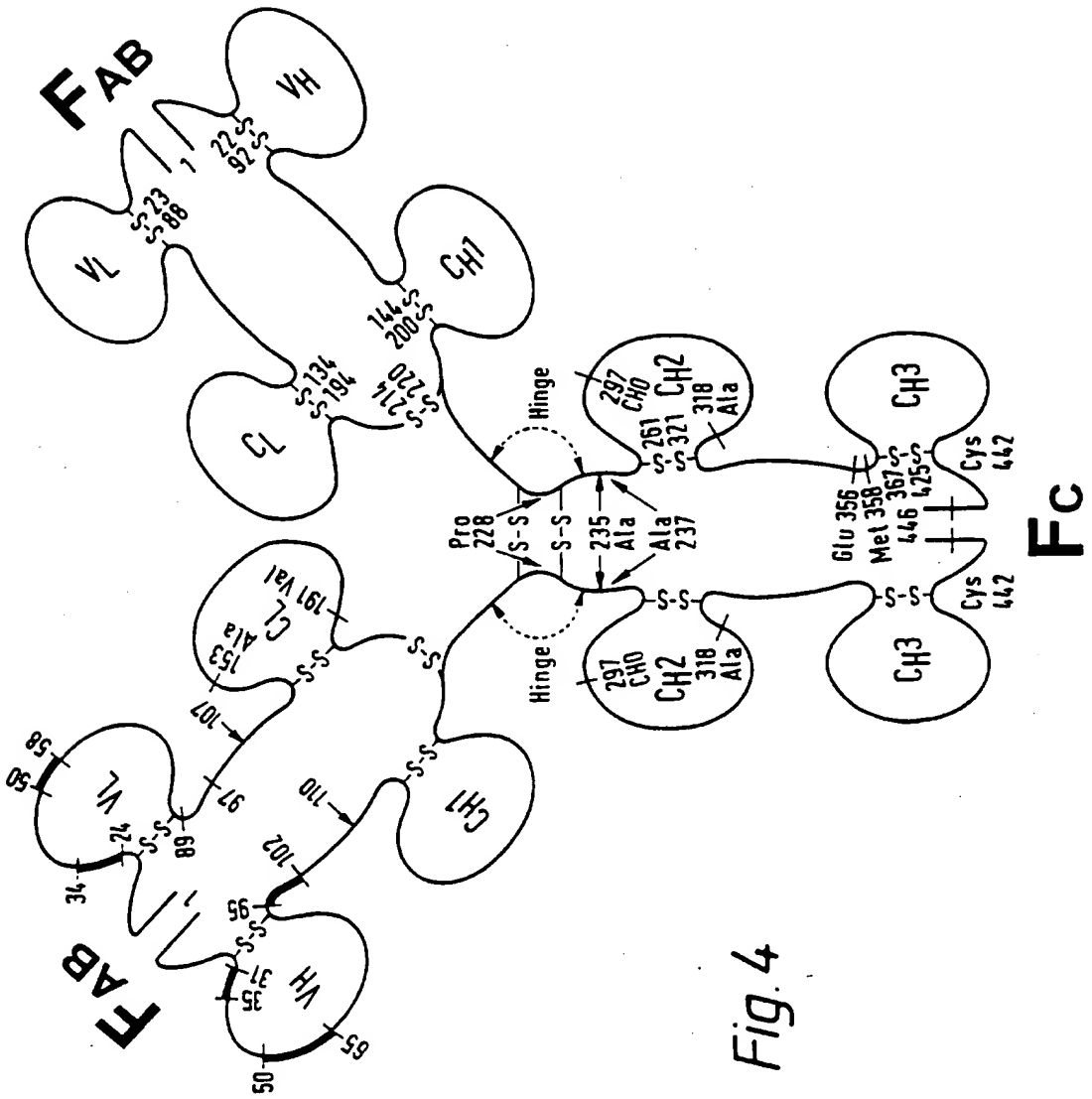

Fig. 4

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 95/02585

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/00 C07K16/30 A61K47/48 A61K51/10 //C12N15/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 19515 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 26 December 1991 see page 3, line 22 - page 4, line 2; claims 1-4	1-4,7,8
Y	see page 7, line 24 - page 8, line 25 see page 9, line 33 - line 35	6,9-24
X	JOURNAL OF IMMUNOLOGY, vol. 148, no. 9, 1 May 1992 BALTIMORE US, pages 2918-2922, B. SHOPES 'A GENETICALLY ENGINEERED HUMAN IgG MUTANT WITH ENHANCED CYTOLYTIC ACTIVITY.' cited in the application	1-4,7,8
Y	see page 2919, left column, line 56 - right column, line 17	6,9-24

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 February 1996	15.03.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Ryckebosch, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCI/GB 95/02585

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROTEIN ENGINEERING, vol. 3, no. 8, 1990 ENGLAND GB, pages 703-708, A. LYONS ET AL. 'SITE-SPECIFIC ATTACHMENT TO RECOMBINANT ANTIBODIES VIA INTRODUCED SURFACE CYSTEINE RESIDUES.' cited in the application	1-3,7,8
Y	see the whole document	6,9-24
X	WO,A,89 01782 (CELLTECH LIMITED) 9 March 1989 cited in the application	1-3,7,8
Y	see the whole document	6,9-24
Y	FEBS LETTERS, vol. 317, no. 1,2, February 1993 AMSTERDAM NL, pages 143-146, A. TOMASSETTI ET AL. 'ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF THE SOLUBLE AND MEMBRANE FORMS OF FOLATE BINDING PROTEIN EXPRESSED IN THE OVARIAN CARCINOMA CELL LINE IGROV1.' cited in the application see page 143, left column, line 1 - right column, line 8	6
Y	EP,A,0 385 601 (CELLTECH LIMITED) 5 September 1990 see page 2, line 48 - page 5, line 37 see page 6, line 5 - line 41 see page 7, line 46 - line 52; claims	9-14,19, 20,22-24
Y	EP,A,0 415 731 (THE WELLCOME FOUNDATION LIMITED) 6 March 1991 cited in the application see claims	15-19, 21,23,24

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB95/02585

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 19-22
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 19-22 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/ composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/GB 95/02585

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